LACTIC ACID BACTERIA TRANSFORMED TO BE PROVIDED WITH A RESPIRATORY METABOLISM

The present invention relates to the enhancing of the preservation and acidification properties of lactic starter culture.

The expression "lactic starter culture" denotes any preparation intended for inoculating a medium to be fermented, and comprising at least one strain of lactic acid bacteria belonging in particular to one of the genera *Lactococcus*, *Streptococcus*, *Enterococcus*, *Leuconostoc*, *Lactobacillus*, *Propionibacteria*, or *Bifidobacteria*, or a mixture of strains belonging to one or more of the abovementioned genera.

The lactic starter cultures used in particular to produce fermented foods and silage products are usually prepared in batch culture, and are then concentrated and packaged for subsequent use so as to inoculate various food products for the purpose of their fermentation. One of the concerns of producers of starter cultures is to obtain a large bacterial biomass, and to maintain good viability of the bacteria during storage so that, during inoculation, the fermentation starts rapidly and gives food products possessing reproducible characteristics.

However, numerous causes of stress can occur during the different stages of preparation of starter cultures and impair the survival of the lactic acid bacteria. In particular, bacterial viability can be rapidly lost if the cultures are maintained in stationary phase. One of the causes thereof is the accumulation, in the medium, of natural products of bacterial metabolism, in particular organic acids such as lactic acid, which cause a reduction in pH which is damaging to bacterial growth. Another cause of loss of viability during

preparation and storage is the presence of oxygen, which is naturally toxic to lactic acid bacteria; these bacteria indeed have in common fermentation-based carbohydrate metabolism. BERGEY's manual, 9th Edition, published by HOLT et al. (1994) WILLIAMS and WILKINS Eds.

To limit the reduction in pH, culture media buffered at around pH 6 with cations combined with carbonates, hydroxides, phosphates or oxides are normally used for the production of lactic starter cultures. However, these additions to the culture medium can cause problems for subsequent productions, for example by promoting the development of phages, or by increasing the solubility of caseins.

To avoid the toxic effects of oxygen, the preparation and storage of starter cultures are normally carried out under anaerobic conditions; for example, during the preparation of batch cultures of starter cultures, some stages are carried out under nitrogen in order to eliminate traces of oxygen. However, during the use of starter cultures, the latter are frequently exposed to high levels of oxygen. For example, the milk which is used for the preparation of fermented dairy products is highly aerated during the transfer processes and is therefore high in oxygen. This could constitute a cause for the slowing down of the restarting of the starter cultures.

It has been reported [A.K. SIJPESTEIJN, *Antonie von Leeuwenhoek* 36: 335, 1970] that *Lactococcus* and *Leuconostoc* cultured in the presence of heme and under aeration produce cytochromes and possess a respiratory metabolism.

More recent studies [KANEKO et al. Appl. Environ. Microbiol., 56: 9, 2644-2649 (1990)] report an improvement in the proliferation of a strain of

Lactococcus lactis diacetylactis, cultured in the presence of hemin and/or Cu^{2+}. These authors did not attribute this effect to the appearance of a respiratory metabolism, but to the activation of diacetyl synthase by hemin and/or Cu^{2+}, which would preferentially orient the fermentative metabolism toward the production of diacetyl, at the expense of lactate.

The Inventors' team has recently discovered that, in the context of the preparation of lactic starter cultures, the use of a porphyrin compound combined with an anaerobic culture made it possible to obtain greater bacterial growth than that obtained during conventional procedures, and that, in addition, the percentage of viable bacteria in the bacteria population and the survival time were also much higher. Furthermore, when the starter cultures obtained in this manner are used to inoculate a product to be fermented, a very rapid restarting of growth and of bacterial fermentation is observed, resulting in a much more rapid acidification of the product than that observed with conventional starter cultures. These studies are described in international application PCT/IB99/01430 (PCT WO 00/05342) in the name of INRA.

The Inventors have now shown that the improvements in bacterial yield and viability during storage were due to the acquisition of a respiratory metabolism by *L. lactis* during the culture under aeration and in the presence of a porphyrin compound. During the inoculation of the product to be fermented, the bacteria are, in addition, capable of immediately restoring a fermentative metabolism, which results in an increase in the restarting performance.

Respiratory metabolism requires the presence of the enzymatic package involved in various metabolic pathways, in particular the synthesis and the use of

heme, the synthesis of cytochromes, and probably the synthesis of at least part of the tricarboxylic acid cycle (Krebs cycle).

Using primers derived from the alignment of sequences of genes known to be involved in respiration in other bacteria, the Inventors have searched for the presence of homologous genes in *L. lactis*. They have thus identified three genes encoding ferrochelatase (enzyme involved in the biosynthesis of heme by catalyzing the formation of a complex between iron and a porphyrin compound which is a precursor of heme, it being possible for the complex thus formed to be incorporated into bacterial cytochromes), cytochrome D oxidase (a hemoprotein necessary for respiration), and aconitase (enzyme involved in the Krebs cycle), respectively.

They have, in addition, shown that the genes encoding cytochrome D oxidase and ferrochelatase were functional in *L. lactis*. They indeed observed that bacteria in which the gene for cytochrome D oxidase is inactive no longer exhibit respiratory metabolism when they are cultured under aerobic conditions and in the presence of an iron-containing porphyrin compound. Similarly, they observed that the inactivation of the gene encoding ferrochelatase resulted in the loss of the respiratory metabolism capacities of *L. lactis* in the case of cultures carried out in the presence of a porphyrin compound not containing iron, such as protoporphyrin, but not in the case of cultures carried out in the presence of an iron-containing porphyrin compound such as heme.

These observations confirm that the improvement in the performance of lactic starter cultures which is obtained by preparing these starter cultures under aerobic conditions and in the presence of a porphyrin compound, described in application PCT/IB99/01430, is linked to the appearance of a respiratory metabolism

under these culture conditions.

The results of the inventors demonstrate in particular that lactic acid bacteria, represented by *L. lactis*, possess the capacity to grow via a fermentative or respiratory mechanism.

The method of growth depends on the culture conditions, but also on the signals transmitted by the cell itself. The metabolism appears to be rather fermentative at the beginning of growth, and then becomes respiratory once the bacteria are in late exporiential growth. The Inventors have, in addition, made the hypothesis that regulation of this transition, exerted by the bacterium, existed and that impairment of this regulation, brought about by the inactivation or by the overexpression of a regulatory gene which controls the transition between fermentative and respiratory growth, can have, as a result, a more effective respiration.

The aim of the present invention is to provide means of conferring a respiratory metabolism on lactic acid bacteria, or of promoting it, in particular so as to improve the performance of lactic starter cultures in a manner comparable to that previously observed during the addition of heme (or of other molecules derived from porphyrins).

In accordance with the present invention, this aim can be achieved by causing or by promoting the expression, in a lactic acid bacterium, of at least one protein participating in this metabolism, by modifying the genomic profile of the lactic acid bacterium, either by transferring, to a lactic acid bacterium, one or more genes for respiratory metabolism, cloned from an aerobic bacterium, or by inactivating or overexpressing a gene of the initial bacterium, so as to tip the metabolism toward the respiratory pathway.

The subject of the present invention is a lactic acid

bacterium which has been genetically modified so as to be provided with a respiratory metabolism, or to activate said metabolism.

This encompasses in particular any lactic acid bacterium which has undergone at least one modification consisting in the addition of at least one gene encoding a protein involved in respiratory metabolism or promoting said metabolism, and/or at least one modification resulting in the activation of at least one protein involved in respiratory metabolism or promoting said metabolism, and/or at least one modification resulting in the overexpression of at least one gene encoding a protein involved in respiratory metabolism or promoting said metabolism, and/or at least one modification resulting in the complete or partial inactivation of at least one gene encoding a protein involved in fermentative metabolism or promoting said metabolism, and/or at least one modification resulting in the underexpression of at least one gene encoding a protein involved in fermentative metabolism or promoting said metabolism.

A modification resulting in the addition of at least one gene encoding a protein involved in respiratory metabolism, or promoting it, may be obtained by transforming said lactic acid bacterium with at least one heterologous gene (that is to say a gene which is not naturally present in said bacterium) which is involved in respiratory metabolism. This may include, in particular, a gene derived from an aerobic bacterium and involved in respiratory metabolism. Said gene may in particular be chosen from,

- the genes encoding proteins of the heme biosynthesis pathway;
- the genes encoding proteins of the cytochrome biosynthesis pathway;
- the genes encoding hemin proteins;
- the genes encoding proteins of the Krebs cycle.

A modification resulting in the overactivation of a protein involved in respiratory metabolism or promoting it may, for example, be obtained by introducing, into the gene encoding this protein, a mutation resulting in a more active protein. A modification resulting in the overexpression of at least one gene encoding a protein involved in respiratory metabolism, or promoting it, may for example be obtained by transforming said lactic acid bacterium with at least one additional copy of said gene, and/or by acting on the *cis* or *trans* regulation of this gene, for example by placing said gene under the control of elements for regulation of expression allowing greater expression (for example strong promoter, constitutive promoter, transcription enhancer, and the like) and/or by inactivating elements for negative regulation of expression which are combined with said gene (repressor, attenuator, and the like).

For example, it is thus possible to overactivate and/or overexpress one or more genes chosen from:

- genes regulating metabolic pathways promoting the respiratory pathway;
- enzymes of the cytochrome biosynthesis pathway;
- genes encoding hemin proteins.

A modification resulting in the complete or partial inactivation of at least one gene encoding a protein involved in fermentative metabolism, or promoting it, may be obtained in particular by deleting all or part of said gene, or by introducing a mutation resulting in the production of a less active or an inactive protein. A modification resulting in the underexpression of at least one gene encoding a protein involved in fermentative metabolism, or promoting it, may for example be obtained by acting on the *cis* or *trans* regulation of this gene, for example by placing said gene under the control of elements for negative

regulation of expression (for example repressor, attenuator, and the like) and/or by partially or completely inactivating the elements for positive regulation of expression (for example promoter, transcription enhancer, and the like) combined with said gene, or by making this expression inducible.

By way of nonlimiting example of activation or overexpression of a gene encoding a protein involved in respiratory metabolism, or promoting it, there may be mentioned in particular:

- an activation of one or more genes involved in the assimilation of heme, or a modification increasing the expression of said gene(s) and/or making it constitutive.

This allows the production of a strain having an earlier and/or a more effective assimilation of heme, which is desirable in cases where the availability of heme constitutes a limiting step for a respiratory metabolism.

In this case, the switch between a fermentative metabolism and a respiratory metabolism can be controlled by modifying the oxygen content of the culture medium and by the presence of heme.

Nonlimiting examples of inactivation or underexpression of genes encoding proteins involved in fermentative metabolism, or promoting it, are in particular:

- an inactivation of the *ccpA* gene, or a modification attenuating its expression or making it inducible. The *ccpA* gene regulates the expression of several genes involved in the catabolism of sugars [LUESINK et al., Molecular Microbiology 30: 789-798, (1998)]. The inventors made the hypothesis that its inactivation could promote the expression of the genes necessary for respiration;

- an inactivation of the *gls24* gene or a modification attenuating its expression or making it inducible. A study in *Enterococcus faecalis* describes the *gls24* gene, which represses the expression of the genes encoding L-lactate dehydrogenase, lipoamide dehydrogenase, pyruvate decarboxylase, all of which are involved in metabolism [GIARD *et al.* J. Bacteriol. 182: 4512-4520, (2000)]. A gene analogous to *gls24* exists in *L. lactis*. The inventors made the hypothesis that a mutant of *L. lactis* where *gls24* is inactive could be advantaged with respect to respiration.

According to a preferred embodiment of the present invention, said lactic acid bacterium is chosen from bacteria of the genera *Lactococcus, Streptococcus, Enterococcus, Leuconostoc, Lactobacillus, Propionibacteria,* or *Bifidobacteria*. Preferred bacteria are those of the different species of the genus *Lactococcus*, as well as streptococci of the species *Streptococcus thermophilus*.

For a given bacterial species, the appropriate gene(s) for conferring on the bacteria all or part of the enzymatic package necessary for the acquisition of a respiratory metabolism or for enhancing respiration may be, for some genes, identified by persons skilled in the art from the information on the sequences of the bacterial genomes available on databases, which makes it possible to identify the genes already present in a given microorganism and the metabolic pathways in which these genes may participate. In the absence of the genomic sequence of the bacterial species of interest, the sequence(s) of one or more related species can be used to determine which genes are probably present. For example, the complete or practically complete sequences of the genome of several species of streptococci (*Streptococcus mutans* and *Enterococcus* [previously *Streptococcus*] *faecalis*) as well as other Gram-positive

bacteria are currently available, and reveal the presence of several of the genes required for respiration. These species are phylogenetically fairly closely related to lactic acid bacteria, commonly used in the food industry, such as thermophilic streptococci, and are also related to lactococci.

Thus, the transformation of bacteria of the species *Lactococcus* or *Streptococcus* with one or more genes encoding one or more proteins of the heme biosynthesis pathway can make it possible to obtain bacteria possessing a respiratory metabolism without the need to add porphyrin derivatives to the culture medium.

The desired genes may be obtained from a strict aerobic bacerium, or from a facultative aerobic bacterium. They can be easily identified from the bacterial genomes available on databases. For example, it is possible to use genes obtained from *Bacillus subtilis*, which is an aerobic bacterium, and whose complete genomic sequence has been published.

It is thus possible to provide a lactic acid bacterium with all the genes necessary to confer a respiratory metabolism. It is also possible, if desired, to provide only a portion of these genes, for example so as to be able to control, in different ways, the conditions under which the bacterium would be capable of respiration.

It is thus possible to construct, by way of nonlimiting examples:

- a lactic acid bacterium possessing all the genes necessary for respiratory metabolism; the switch between a fermentative metabolism and a respiratory metabolism may be controlled by modifying the oxygen content of the culture medium;
- a lactic acid bacterium possessing all the genes

encoding the Krebs cycle proteins and all the cytochrome genes, but lacking all or some of the genes of the heme biosynthesis pathway; the switch from a fermentative metabolism to a respiratory metabolism will then require, in addition to the aeration of the medium, the addition of heme or of one of its precursors.

For the production of a transformed lactic acid bacterium in accordance with the invention, the desired gene(s) may be introduced separately, or at least some of them may be grouped into one or more operons.

For example, to provide *L. lactis* with the total or partial capacity to biosynthesize heme, one or both *B. subtilis* heme operons or only some of the genes present on these operons may be transferred into *L. lactis*.

To obtain lactic acid bacteria in accordance with the invention, it is also possible to promote the expression of genes involved in respiratory metabolism which are already naturally present in said bacteria. This may be carried out, for example, by acting on the *cis* or *trans* regulation of the activity of these genes.

In addition to the abovementioned genetic modifications, which make it possible to provide them with a respiratory metabolism, or to promote said metabolism, the lactic acid bacteria in accordance with the invention may comprise, in addition to other modifications, in particular the introduction of one or more nucleic acid sequences allowing them produce substances of interest.

Lactic acid bacteria in accordance with the invention may be obtained using conventional genetic engineering techniques known per se to persons skilled in the art.

For the cloning of the genes, the desired gene(s) may

be combined with sequences for the control of transcription and of translation which are functional in the lactic acid bacterium which it is desired to transform. It is possible in particular, if desired, to place one or more of the transferred genes under transcriptional control of an inducible promoter, in order to allow control of the switch between fermentative metabolism and respiratory metabolism.

The constructs prepared are placed in an appropriate vector in order to introduce them into the relevant lactic acid bacterium. Vectors which can be used for transforming lactic acid bacteria of different species, and which make it possible either to maintain the genetic information introduced in the form of a stable independent replicon, or to integrate it into the bacterial chromosome, are known per se. The integration into the bacterial chromosome may be carried out by transposition, or by a method for replacing genes by homologous recombination, according to methods known per se to persons skilled in the art. By way of nonlimiting examples of methods which can be used and of vectors allowing the implementation of these methods, there may be mentioned in particular the methods and vectors described in PCT application WO 93/18164 in the name of INRA.

In the cases where the quantity of genetic information to be transferred requires the introduction of large segments of DNA, it is possible to use protoplast fusion or bacterial conjugation techniques.

Lactic acid bacteria in accordance with the invention may also be produced by selection of mutants, which are natural or which are obtained by random mutagenesis, in which the activity and/or the expression of a protein involved in fermentative metabolism, or promoting it, is reduced or nonexistant, or from the selection of mutants in which the activity and/or the expression of

a protein involved in respiratory metabolism is increased.

The functioning of respiratory metabolism in the modified bacterium in accordance with the invention may be checked by culturing said bacterium under conditions allowing the induction of a respiratory metabolism (that is to say under aeration, and optionally under conditions for inducing one or more inducible promoters optionally controlling the expression of one or more of the transferred genes and/or in the presence of heme or of one or its precursors in the case where the transformed bacterium does not contain all the genes of the heme biosynthesis pathway, and the like), and by measuring the following parameters: i) the pH of the final culture, ii) the products consumed or formed during the period of culture (for example the oxygen consumed, the production of fumarate or that of lactate, the total quantity of carbon at the end of culture, which makes it possible in particular to evaluate the production of CO_2 during respiration, and the like), iii) the bacterial population at the end of growth, iv) the survival during long storage, and v) the reacidification properties when the transformed strain is used as starter (starter culture) for fermentation. If desired, a detection of heme in the cells, or of the activity of the proteins requiring heme to function (such as, for example, cytochromes), may be carried out.

Modified strains of lactic acid bacteria in accordance with the invention, when they are cultured under aerobic conditions, exhibit substantial growth, which makes it possible to propose their use as host cells in the context of conventional methods for producing substances of interest by genetic engineering.

The object of the present invention is also a method for culturing lactic acid bacteria, characterized in

that it comprises the culture of at least one strain of lactic acid bacterium in accordance with the invention under conditions allowing the induction of a respiratory metabolism in said strain.

Said conditions for inducing respiratory metabolism comprise aeration of the culture; advantageously, this aeration is carried out so as to maintain, during the entire period of culture, an oxygen supply equal to at least 5 millimoles per liter of culture medium.

According to the characteristics of the strain in accordance with the invention which is used, and in particular according to its capacity to assimilate heme, or to carry out the biosynthesis thereof, said conditions for inducing respiratory metabolism may also comprise the addition of a porphyrin derivative to the culture medium as described in application PCT/IB99/01430.

Most advantageously, the strains of lactic acid bacteria in accordance with the invention may be used for the production of lactic starter cultures.

In this case, the method in accordance with the invention comprises, in addition, the harvesting of the bacteria at the end of said culture, and, optionally, their packaging and their storage, by any appropriate means.

The bacteria may be harvested by any means known per se; it is possible, for example, to distribute the culture into appropriate packaging and to preserve it in this form up to the time of use; generally, it will be preferable, however, to separate the bacteria from the culture medium and to concentrate them by centrifugation or by filtration. The harvested bacteria can then be packaged with a view of their preservation.

The present invention also encompasses the lactic starter cultures comprising at least one modified strain of lactic acid bacterium in accordance with the invention, and in particular starter cultures which can be obtained by the method in accordance with the invention.

These starter cultures may also comprise one or more other bacterial strains, of the same species or of different species. Several different species or several different strains may have been cultured simultaneously (in the case where their optimum growth conditions are compatible), or cultured separately and combined after harvest.

The lactic starter cultures in accordance with the invention may be harvested and preserved under the same conditions as the lactic starter cultures of the prior art, and in particular as the lactic starter cultures which are the subject of application PCT/IB99/01430; they possess preservation and restarting properties which are at least comparable to those of the latter.

The invention also encompasses the use of the lactic starter cultures in accordance with the invention for the production of fermented products. In particular, the subject of the invention is a method for preparing a fermented product, characterized in that it comprises the inoculation of a medium to be fermented using a lactic starter culture in accordance with the invention.

The invention will be further illustrated using the additional description which follows, which refers to nonlimiting examples of obtaining lactic acid bacteria in accordance with the invention.

EXAMPLE 1: PRODUCTION OF A STRAIN OF *L. LACTIS* EXPRESSING THE GENES NECESSARY FOR PRODUCING PROTOHEME

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hemA gene (NADP(H):glutamyl-tRNA reductase, SWISS-PROT accession number: P16616), *hemL* gene (GSA 2,1-aminotransferase, SWISS-PROT accession number: P30949), *hemB* gene (porphobilinogen synthase, SWISS-PROT accession number: P30950), *hemC* gene (hydroxymethylbilane synthase, SWISS-PROT accession number: P16616), *hemD* gene (uroporphyrinogen III synthase, SWISS-PROT accession number: P21248), and *hemE* gene (uroporphyrinogen decarboxylase, SWISS-PROT accession number: P32395), *hemY* gene (coproporphyrinogen III oxidase and protoporphyrinogen oxidase functions, SWISS-PROT accession number: P32397) and *hemH* gene (ferrochelatase, SWISS-PROT accession number: P32396) of *Bacillus subtilis* allow the synthesis of protoheme IX from glutamyl-tRNA.

The *hemACDBL* genes contained in a single operon in *B. subtilis* are amplified by PCR from the strain 3G18 (pLUG1301) [HANSSON AND HEDERSTEDT, J. Bacteriol., 174(24): 8081, (1992)] using primers which make it possible to obtain the coding sequence of the genes with the promoter, the ribosome-binding site and the terminator:

Sense primer:

5'-GGGGAGCTCGGTATTGTCAATAGGAATGC-3'.

Antisense primer:

5'-GGGGATCCGTGGGAGAGCACGAAAAA-3'.

The amplification [5 min 96°C, (30 s 96°C, 1 min 55°C, 5 min 72°C) 30 times] is carried out with 5 units of high fidelity Taq polymerase (promega) in the presence of 4 mM of $MgCl_2$.

A fragment of 6 500 bp is obtained. This fragment is then cloned into the plasmid pCR-TOPO (INVITROGEN) in the *E. coli* TOP10 strain (INVITROGEN). The plasmid obtained, called pTHem1, is digested with *Spe*I and

treated with DNA polymerase, Klenow fragment, in order to obtain a blunt end. The plasmid pTHem1 is then digested with *Sac*I and the *hemAXCDBL* fragment is purified. It is integrated into the plasmid pIL252 previously digested with XhoI, treated with Klenow and then with SacI [SIMON AND CHOPIN, Biochimie, 70: 559-566, (1988)]. The resulting plasmid, called pILHem1, is introduced into the *L. lactis* MG1363 strain [GASSON, J. Bacteriol., 154: 1-9, (1983)]. The production of uroporphyrinogen III by this strain is determined as previously described by ANDERSON and IVANOVICS, (J. Gen. Microbiol., 49: 31-40, (1967)].

The *hemEHY* genes contained in a single operon in *B. subtilis* are amplified by PCR from the strain 3G18 (pLUG1301) using primers which make it possible to obtain the coding sequence of the genes with or without the promoter, with the ribosome-binding site and the terminator:
sense primer:
5'-GGGATCCGTATGAAAGGTGGAAATC-3', without promoter
5'-GGGGGATCCGGCGATTTTTTGAACTTTGAGCTACA-3', with promoter
antisense primer:
5'-GGGCTCGAGACACAATATTGCCATTGCACATC-3'.

The amplification [5 min 96°C, (30 s 96°C, 1 min 55°C, 5 min 72°C) 30 times] is carried out with 5 units of high fidelity Taq polymerase (promega) in the presence of 4 mM of $MgCl_2$. A fragment of 3 600 bp is obtained. This fragment proves to be unclonable into the cloning systems used in *E. coli* or in *L. lactis*. This may be due, according to the literature, to the toxicity of the product of the *hemY* gene in *E. coli*. By extension, it is not impossible that HemY could also be toxic in *L. lactis*.

The *hemEH* genes contained in the *hemEHY* operon in *B. subtilis* are amplified by PCR from the strain 3G18 (pLUG1301) using primers which make it possible to

obtain the coding sequence of the genes with the ribosome-binding site.

Sense primer:

5'-GGGGTACCTCTAGACCGTATGAAAGGTGGAAATCAG-3'

Antisense primer:

5'-CCATCGATCTTTAACGTCCTAATTTTTTTAATAC

This fragment is then cloned into the plasmid pCR-TOPO (INVITROGEN) in the *E. coli* TOP10 strain (INVITROGEN). The plasmid obtained, called pTHem4, is linearized with *Xba*I, and then the ends are made blunt by the Klenow fragment of DNA polymerase. The plasmid pTHem4 is then digested with *Cla*I and the *hemEH* fragment is purified. This fragment is then placed under the control of the promoter P_{nis}, which is inducible with nisin (NICE system, patent EP0712935 by VOS and KUIPERS), in a plasmid derived from pNZ8020 previously cleaved with *Bam*HI, treated with Klenow polymerase, and then cleaved with *Cla*I. The resulting plasmid, called pGHem1, is introduced into the *L. lactis* NZ9000 strain containing the plasmid pILHem1. The production of protoheme IX by this strain is determined as described by SHIBATA, [Methods of biochemical analysis, D. Glick (Ed.), Interscience, New York, Vol. VII, 77-109, (1959)].

The *hemACDBL* and *hemEH* operons are used to complement corresponding mutants of *B. subtilis* (Bacillus Genetic Stock Center) so as to ensure their functionality.

The strains obtained are tested for their capacity for autonomous respiration under aeration culture conditions, with or without hemin and by inducing the expression of the *hemEH* operon with nisin. The strain used as negative control is a strain NZ9000 containing the vector plasmids alone pIL252 and pGK:CmR:P_{nis}, respectively. The optical density of the cultures is monitored at 600 nm. Under aeration condition, with hemin, the OD_{600} values obtained are 2.87 for the negative control and 3.23 for the strain containing the

hem genes. Without hemin, the values are 2.10 for the negative control and 2.78 for the strain containing the *hem* genes. These results show that the introduction of the *B. subtilis hemA, hemL, hemB, hemC, hemD, hemE* and *hemH* genes into *L. lactis* seems sufficient to bring about the biosynthesis of heme and lead to a partial respiratory phenotype.

The results obtained during the monitoring of the growth of the *L. lactis* strain containing the *B. subtilis hemA, hemL, hemB, hemC, hemD, hemE* and *hemH* genes, compared with a control strain, are summarized in table I below.

Table I

Culture conditions	Optical density A_{600} (biomass)	
	Control (without *hem* genes): *L.lactis* NZ9000 (pIL252:pGK:CmR:p_{nis})	Cloned *hem* genes: *L. lactis* NZ9000 (pILHem1:pGHem1)
Aeration, nisin without hemin	2.1	2.8
Aeration, nisin plus hemin	2.9	3.2

EXAMPLE 2: SCREENING FOR THE ISOLATION OF AN *L. LACTIS* STRAIN HAVING A BETTER RESPIRATORY CAPACITY

The respiration of *L. lactis* depends in particular on the capacity of the cell to assimilate hemin, an essential cofactor for the respiratory activity. According to the studies by KAY *et al.* [J. Bacteriol. 164: 1332-1336, (1985)] and ISHIGURO *et al.* [J. Bacteriol. 164: 1233-1237, (1985)], bacteria which accumulate hemin are also capable of binding a dye, Congo red. The use of Congo red makes it possible to isolate strains binding the dye to greater or lesser degree than the control.

Random mutagenesis is carried out on the *L. lactis*

MG1363 strain according to the procedure by MAGUIN et al. [J. Bacteriol., 178: 931-935, (1996)]. The cells are plated on a dish containing Congo red at 30 µg/ml. The mother strain is used as control.

White mutants (binding the dye less) are isolated. These mutants assimilate hemin less efficiently than the control and are respiration deficient.

Mutants which are redder than the control are also isolated. These mutants, which find it easier to isolate hemin, will be potentially more capable of respiration than the control.

The mutated genes may then be identified by techniques known to persons skilled in the art, for example according to the procedure by MAGUIN *et al.* [J. Bacteriol., 178: 931-935, (1996)].

EXAMPLE 3: PRODUCTION OF AN *L. LACTIS* STRAIN EXPRESSING THE GENES REQUIRED TO PRODUCE QUINONES

The addition of Vitamin B2 (riboflavin) to the M17 glucose medium can stimulate respiration in *L. lactis* MG1363 (increase in the biomass). For this purpose, it is possible to increase the biomass undergoing respiration by the production of Vitamin K2 (menaquinone). This vitamin is also an essential component of the respiratory chains. Based on the chromosomal sequence of IL1403, which is close to MG1363, it is observed that some genes are absent in relation to what is known in Gram-positive bacteria (*Bacillus subtilis*).

The cloning of the *Bacillus subtilis men* operon into *L. lactis* can therefore promote respiration in the latter.

The *menFBytxMmenBEC* operon comprises five genes (*Bacillus*

subtilis and other Gram-positive bacteria, Ed. Sonenshein, A.L., Hoch J.A. and Losick R., ASM, W. DC):

menF: menaquinone-specific 2-ketoglutarate dehydrogenase, SWISS-PROT accession number 23973

menD: 2-succinyl-6-hydroxy-2,4-cyclohexadiene-1-carboxylate synthase, SWISS-PROT accession number 23970

menB: 1,4-dihydroxy-2-naphthoic acid synthase, SWISS-PROT accession number 23966

menE: o-succinylbenzoic acid coenzyme A synthase, SWISS-PROT accession number 23971

menC: o-succinylbenzoic acid synthase, no SWISS-PROT accession number.

The genes are amplified by PCR from the strain 168 [ANAGNOSTOPOULOS *et al.*, J. Bacteriol. 81: 741-747, (1961)] using primers which make it possible to obtain the coding sequence of the genes with the promoter and its terminator.

Sense primer:

5' GTACTGCTGCCATCAGCCC 3'

Antisense primer:

5' CCACGTCCTGTGACGAATACTCCGC 3'

The fragment of about 8 kilobases is cloned into a multicopy plasmid of the pIL253 type (SIMON *et al.* Biochimie 559-566 1988). The functionality of the genes is determined by complementation of *men* mutants in *B. subtilis* [MILLER *et al.*, J. Bacteriol., 170: 2735-2741, (1988)]. The production of quinone is determined according to the procedure by MORISHITA *et al.*, [J. Diary. Sci. 82: 1879-1903, (1999)].

EXAMPLE 4: ISOLATION OF THE MUTANT STRAINS OF *L. LACTIS* HAVING A BETTER RESPIRATORY CAPACITY

The respiratory capacity is characterized by the presence of heme in the cell. The gene encoding catalase of a *Bacillus subtilis* strain has been previously cloned into *L. lactis* (application

PCT/FR00/00885 in the names of INRA and CEA; Inventors BRAVARD and DUWAT). Catalase requires heme for its activity. Into the *L. lactis* strain containing the cloned gene for catalase, a tool for transposition, pGhost9:ISS1 (PCT application WO 94/18164), is introduced. Mutagenesis is performed and the mutants derived from the mutagenesis are screened for their catalase activity in the presence of a small quantity of hemin. The colonies demonstrating a high catalase activity are selected. The mutation responsible for the increase in the catalase activity is identified by techniques known to persons skilled in the art, for example according to the procedure by MAGUIN *et al.*, [J. Bacteriol., 178: 931-935, (1996)]. The respiratory activity is tested for all the mutant strains having an increased catalase activity compared with the wild-type strain. Among the mutant strains, those having a more efficient respiration can be identified by an increase in biomass, high final pH, and/or respiration in the presence of a smaller quantity of hemin. Mutants will be reconstructed, and/or the plasmid containing catalase can be eliminated.

EXAMPLE 5: PRODUCTION OF AN *L. LACTIS* STRAIN WHOSE METABOLISM IS DIVERTED TO RESPIRATION

The enzymes which catalyze the degradation of sugars for the production of energy are under the control of regulators. The regulator CcpA regulates the expression of several glycolitic enzymes, including phosphofructokinase, pyruvate kinase and L-lactate dehydrogenase. A *ccpA* mutant, characterized under fermentation conditions, produces a reduced quantity of lactate, but a larger quantity of acetate and of ethanol, which confirms the regulatory role of CcpA (LUESINK *et al.*, Molecular Microbiology 30: 789-798, (1998)]. No previous work describes the behavior of a *ccpA* mutant of lactic acid bacteria under respiratory conditions. The inventors made the hypothesis that a

ccpA mutant could adopt a respiratory metabolism from the start of the culture, thus improving biomass acquisition.

The respiratory capacity of a strain carrying a mutation in the *ccpA* gene is tested. *ccpA* mutants are obtained either by gene replacement [LUESINK *et al.*, Mol. Microbiol. 30: 789-798, (1998)], or by transposon insertion.

The strain used in this example (described by ALEKSANDRZAK *et al.*, Food Biotechnology 17: 61-66, 2000) contains a *ccpA* gene inactivated by the insertion of a transposon, (but it is probable that any *ccpA* mutant gives similar results). The growth and the final biomass are determined in M17 plus glucose (1%) medium or BHI plus glucose (1%) medium, and containing or otherwise hemin (10 µg/ml) and aerated or not aerated. The inocula are prepared in M17 glucose.

The results showing the respiratory capacity of the mutant relative to the wild-type strain are illustrated by table II below, and by figure 1. These data indicate that the *ccpA* mutant has, at the end of the culture, a biomass and a pH greater than the wild-type strain.

Table II

Strain	Growth conditions	Optical density A_{600} (biomass)		Final pH	
		M17	BHI	M17	BHI
ccpA (derived from the IL1403 strain)	without aeration	2.3	ND	5.2	ND
	aeration without hemin	2.8	2.7	5.2	4.2
	aeration plus hemin	3.6	5	5.6	5.3
IL1403 (wild type)	without aeration	2.3	ND	5.2	ND
	aeration without hemin	2.6	1.5	5.2	4.2
	aeration plus hemin	3.2	3.9	5.4	4.5

ND: not determined

Figure 1 represents the growth of the *ccpA* mutant,

relative to that of the wild-type strain IL1403, in BHI medium containing 1% of glucose, and under aeration conditions, in the presence or in the absence of hemin. Symbols: ■, *ccpA* + hemin; □, *ccpA* without hemin; ◆, IL1403 + hemin; Δ, IL1403 without hemin.

For the preparation of starter cultures, the *ccpA* gene may also be placed under the control of an inducible promoter. The culture of bacteria for the preparation of the starter culture is carried out under conditions which do not induce the promoter, and *ccpA* is not expressed. The use of the starter culture may take place under conditions inducing the promoter, and thus allowing the restoration of the expression of *ccpA*, producing a strain having activities equivalent to those of the wild-type strain.